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運用基因微陣列分析法探討機械張力對心臟細胞基因表現的作用以及相關基因的功能闡釋

cDNA microarray analysis of differential expression of mechanical stretch-related genes and unravel their functional roles in neonatal rat cardiomyocytes

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中文摘要

生物物理壓力如機械張力(mechanical stretch)的作用於心臟細胞，會導致心臟細胞的肥大現象，對臨床及基礎的心臟學研究而言，如何控制心臟細胞肥大現象的發生，是一個具有挑戰性的問題，尤其對發展新的心臟疾病治療策略更為重要。至目前為止，控制心臟細胞肥大現象的發生的機制仍不清楚，而回答此問題的一個方法就是找出控制心臟細胞肥大現象的發生的一系列基因。假如我們能明瞭如何控制心臟細胞肥大現象發生的機制，或許就能抑制心臟細胞肥大現象的發生，甚至後續的細胞凋亡，進而發展出新的心臟衰竭或心臟相關疾病的治療策略。為達此目標，篩選及定性參與心臟細胞肥大現象的發生的關鍵分子是必要的。以機械張力作用於心臟細胞，造成心臟細胞的肥大現象，對於細胞內不同基因表現的影響以及相關基因功能的闡釋尚有許多不明之處。在本研究計劃中，我們將運用基因微陣列（cDNA microarray）分析法探討機械張力對心臟細胞基因表現的作用。基因微陣列為發展技術較成熟且能同時分析大量基因的科技之一，已廣泛應用於許多研究領域以鑑定新的基因。

在本計劃中，我們將以機械張力作用於細胞能誘發心臟細胞肥大的實驗模式，觀察機械張力對心臟細胞的作用，並利用微陣列剖繪其基因表現的型態。將所篩選的基因送入原細胞中並大量表現，研究其對機械張力所誘發心臟細胞肥大的調控作用，配合微陣列可研究其下游受影響的基因為何。為研究基因的調控作用，將選殖啟動子並調查甲基化對細胞肥大的影響。對篩選出的基因，進一步將研究其蛋白質間的交互作用及在細胞中的作用位置，這些努力將提供許多的線索以進一步闡釋心臟細胞肥大的分子機制，並有助於發展新的治療策略。

以基因微陣列分析機械張力對心臟細胞的作用，驗結果顯示：機械張力可以影響心臟細胞內許多基因的表現(表一) ，其中包括增加內皮素型一氧化氮合成酶(endothelial nitric oxide synthase; eNOS)的基因表現，此增加作用可以北方式點墨法及西方式點墨法作進一步的確認，機械張力亦會短暫增加 eNOS 的酵素活性

及一氧化氮的生成，若給予心臟細胞一氧化氮供獻劑如 S-nitroso-N-acetylpenicillamine (SNAP)或 3-morpholinosydnonimine (SIN) 則能抑制機械張力誘發心臟細胞肥大的作用，由此初步結果可知機械張力會短暫增加 eNOS 的基因表現，並於機械張力誘發心臟細胞肥大的過程中扮演保護的作用，進一步的研究工作將探討機械張力對心臟細胞其它基因表現的作用並設計實驗闡釋這些相關基因的生理功能。

關鍵詞： 機械張力 •基因微陣列• 內皮素型一氧化氮合成酶 • 心臟細胞肥大

英文摘要

Mechanical stretch induced by high blood pressure is an initial factor leading to cardiac hypertrophy. However, the effect of mechanical stretch on the modulation of gene expression in cardiomyocytes still remains unclear. A challenging problem in cardiology is how to control the process of cardiomyocyte hypertrophy, which is particularly useful to develop new treatment strategies for heart failure patients. The molecular mechanism(s) responsible for the process of cardiomyocyte hypertrophy is still unclear. One approach to address this challenge is to identify the genetic events of controlling cardiomyocyte hypertrophy. If we can understand the molecular mechanisms on how to control the process of cardiomyocyte hypertrophy, it may facilitate the development of new therapies for cardiac diseases. To achieve this goal, identification and characterization of key molecules participated in cardiomyocyte hypertrophy are essential.

The mechanical stretched-cardiomyocyte is a good model for the study of cardiomyocyte hypertrophy. cDNA microarray method, a powerful tool for massively parallel analysis of gene expression, have been applied in various biological studies for identifying differentially expressed genes. In this project, we will compare the transcript profiles of mechanically-stretched neonatal rat cardiomyocytes with that of untstretched cells by hybridization of cell-derived cDNA to DNA probes immobilized on microarrays. Neonatal rat cardiomyocytes are cultured on malleable silicone dishes and are stretched by 20%. The downstream genes of candidates will also be investigated by microarray. To study gene regulation, we plan cloning the minimal promoter domain and investigating its transcriptional control. We will also characterize candidates' functions involved in the process of cardiomyocyte hypertrophy on protein level and study the protein-protein interactions and the

sub-cellular localization of protein. These efforts may provide the clues to interpret the mechanisms of cardiomyocyte hypertrophy and develop the new therapeutic strategies.

We used the microarrays contained probes for 480 known genes including signal transduction, cell cycle regulators, cytoskeleton and cell motility, and so on. Eighteen genes were identified that showed significantly differential expression in response to one hour of mechanical stretch in cardiomyocytes. Of the represented genes expressed, endothelial nitric oxide synthase (eNOS) gene was the most interesting one. Northern blot and western blot analysis further quantified the expression of eNOS gene. Mechanical stretch also transiently increased constitutive NOS activity and NO production. Furthermore, NO donors such as S-nitroso-N-acetylpenicillamine (SNAP) or 3-morpholinosydnonimine (SIN) inhibited the mechanical stretch-induced cardiomyocyte hypertrophy. The results of this part of study indicate that mechanical stretch transiently induces eNOS gene expression, thereby increasing constitutive NOS activity and NO production, which plays a protective role in the prevention of cardiomyocyte hypertrophy. The fourth going work will be performed to unravel other novel unknown stretch-related genes and their functional roles in cardiomyocytes.

Key Words: mechanical stretch •cDNA microarray •endothelial nitric oxide synthase • cardiomyocyte hypertrophy

第一章 前言

Functional genome analysis is thought to be one of the most promising research projects in twenty-first century after human genome project. Some new and foresighted gene analysis techniques were developed in the latter of the nineties, such as cDNA microarray (Schena et al., 1995; DeRisi et al., 1996; Chen et al., 1998), DNA chip (Fodor , 1997), serial analysis of gene expression (Velculescu et al., 1995; Zhang et al., 1997) and others. In particular, cDNA microarray is the more mature technique among them, and has been applied in various biological studies for identifying differentially expressed genes (Chen et al., 1998; Iyer et al., 1999; Ross et al., 2000; Hong et al., 2000; Chen et al., 2001). Microarray method can be used either for monitoring the expression levels of known genes or for searching differentially expressed genes. Especially after large amounts of expressed sequence tags (ESTs) are published (Boguski et al., 1993; Boguski et al., 1994), it will be even more powerful in its detection of abnormal genes for diseases, e.g., cancer and genetic diseases.

Biomechanical stretch, hypoxia, and other types of stress induce hypertrophy, apoptosis, contractile failure, and other myocardial changes that directly or indirectly predispose to cardiac failure. Until now, the molecular pathways responsible for these changes are only partly understood. Mechanical stretch has been reported to cause an induction of immediate-early genes such as *c-fos* (Komuro et al., 1990), *c-jun*, *c-myc* and *egr-1* (Sadoshima et al., 1992) and increase several gene expressions such as atrial natriuretic peptide (Jarygin et al., 1994), endothelin-1 (Yamazaki et al., 1996) and angiotensinogen gene expression (Shyu et al., 1995a) and regulates myosin heavy chain (MyHC) gene expression (Shyu et al., 1995b) and eventually lead to cell hypertrophy in cardiomyocytes. However, the effect of mechanical stretch on the modulation of gene expression in cardiomyocytes still remains to be further

examined.

In this project, we plan to compare the transcript profiles of mechanically-stretched neonatal rat cardiomyocytes with that of stationary control cells by hybridization of cell-derived cDNA to DNA probes immobilized on microarrays. The preliminary result revealed that endothelial nitric oxide synthase (eNOS) gene was induced by mechanical stretch. The expression of eNOS gene was further quantified by Northern blot and western blot analysis. Mechanical stretch also increased constitutive NOS activity and NO production. Furthermore, NO donors such as S-nitroso-N-acetylpenicillamine (SNAP) or 3-morpholinosydnonimine (SIN) inhibited the mechanical stretch-induced cardiomyocyte hypertrophy. The preliminary result clearly indicate that mechanical stretch transiently induces eNOS gene expression, thereby increasing constitutive NOS activity and NO production, which plays a protective role in the prevention of cardiomyocyte hypertrophy. The fourth going work will be performed to profile the expression of genes involved in the mechanical stretch treatment. Furthermore, the characterization of individual gene selected from above mention as well as the mechanical stretch-related unknown genes will be also carried out in this project.

SPECIFIC AIMS

The goal of this project is to compare the transcript profiles of mechanically-stretched neonatal rat cardiomyocytes with that of stationary control cells by hybridization of cell-derived cDNA to DNA probes immobilized on microarrays. When some of the specifc genes are identified that showed significantly differential expression in response to mechanical stretch in cardiomyocytes, Northern blot and western blot analysis will be further used to quantify the specific gene expression. Some further experiments will be designed and performed to define the function of

these specifc genes.

第二章 材料方法

Materials

Taq DNA polymerase and Superscript II reverse transcriptase polymerase are obtained from GIBCO BRL, a PTC-100 programmable temperature controller is purchased from MJ Research, and RNase H is purchased from MBI Fermentas. Klenow DNA polymerase, oligo(dT), and guanidine thiocyanate are purchased from Pharmacia. A rat eNOS cDNA is kindly provided by Dr. Ten-Nan Lin (Institute of Biomedical Sciencies, Academia Sinica). All other chemicals, of reagent grade, are obtained from Sigma.

Culture of Cardiac Myocytes

Primary cultures of neonatal rat ventricular myocytes are prepared as previously described (Cheng et al., 1999). Briefly, ventricles from 1- to 2-day-old neonatal Sprague-Dawley rats are cut into chunks of approximately 1 mm^3 using scissors and subjected to trypsin (0.125%, GIBCO) digestion in phosphate-buffered saline (PBS). Trypsin-digested cells are collected by centrifugation at 1200 rpm for 5 min. The cell pellet is re-suspended in a medium containing 80% F10 nutrient mixture, 20% fetal calf serum, penicillin (100 units/ml) and streptomycin (100 μg/ml) and plated on a culture dish. The non-attached myocytes in the medium are collected and then plated on the flexible membrane base culture dish with cell density at $1x10^7$ cells/dish. After 2 days in culture, cells are transferred to medium containing 90% DMEM nutrient mixture, 10% fetal calf serum, penicillin (100 units/ml) and streptomycin (100 μg/ml). Myocytes cultures thus obtained were >80% pure as revealed by their contractile characteristics under light microscopy.

In Vitro Cyclic Stretch on Cultured Cardiomyocytes

The stretch unit Flexcell FX-2000 (Flexcell International Co.) consists of a

vacuum unit linked to a valve controlled by a computer program. Cardiomyocytes cultured on the flexible membrane base are subjected to cyclic stretch produced by this computer-controlled application of sinusoidal negative pressure. These flexible membranes supporting cultured cells are stretched by 20% at a frequency of 1 Hz (60 cycles/min) for various time periods. After experiments, culture supernatants, total RNA and total protein from unstrained or strained cells are collected for analysis of NO concentration, NOS activity, mRNA and NOS protein.

Preparation of cDNA probes

The labeling reactions are performed during reverse transcription in the presence of 6 μM random primers or oligo-d$(T)_{20}$VN degenerate primers; 0.5 mM each dATP, dCTP, and dGTP; 40 μM dTTP; 40 μM biotin-16-dUTP (Roche Molecular Biochemicals; Mannheim, Germany); 1X reactio buffer; 10 mM DTT; 0.5 unit/μl Ribonuclease inhibitor (GIBCO-BRL; Gaithersburg, MD); and 200 units of MMLV reverse transcriptase (GIBCO-BRL; Gaithersburg, MD) in a 50-μl solution. The reaction mixture is incubated at room temperature for 10 minutes, then transferred to 42 °C for 90 minutes and is stopped by heating of the reaction mixture to 99 °C for 5 minutes. The RNA is degraded by adding of 5.5 μl of 3 M NaOH followed by a 30-min incubation at 50 °C. The labeled samples are neutralized by addition of 5.5 μl of.3 M acetic acid and then precipitated by adding of 50 μl of 7.5 M ammonium acetate, 20 μg of linear polyacrylamide as carrier, 375 μl of absolute alcohol, and water to make a total of 525 μl. The solution is mixed evenly and stood at –80 °C for 30 min, and then centrifuged at 14,000 rpm for 20 min to precipitate single stranded DNA probe. Pellet is washed with 1 ml of 70 % of ethanol and dried by speed vacuum, then dissolved in 5 μl or suitable volume of deionized water.

Gene microarray hybridization

The membrane carrying the double-stranded cDNA targets is pre-hybridized in 3 ml (option) hybridization buffer (5× SSC, 0.1 % N-lauroylsarcosine, 0.1% SDS, 1% blocking reagent made by Roche Molecular Biochemicals, and 50 μg/ml salmon sperm DNA) at 68°C for 1 hour before hybridization is carried out. cDNA probes (up to 5μg) are resuspended in 100 μl (option) hybridization buffer containing 200 μg/ml $d(A)_{10}$ and 300 ~ 400 μg/ml human COT-1 DNA (GIBCO-BRL) to prevent non-specific binding and are hybridized to the cDNA fragments on the membrane by Southern hybridization procedure. The 100-μl reaction mixture is sealed with the membrane in a hybridization bag or assembly (SureSeal, Hybaid, Middlesex, UK) attached to a weight and incubated at 95°C for 2 min (could be omitted) and then at 68°C for 12 hours. The membrane is then washed with 2× SSC containing 0.1% SDS for 5 min at room temperature followed by three washes with 0.1× SSC containing 0.1% SDS at 65 °C for 15 min each.

Colorimetric detection and image analysis.

After hybridization, the membrane is blocked by 1 ml (option) of 1 % blocking reagent (Roche Molecular Biochemicals) containing 2 % dextran sulphate at room temperature for 1 hour and then is rinsed with 1 X TBS buffer solution (10 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.3% BSA). To detect the spots on the membrane in single-color mode, β-galactosidase-conjugated streptavidin (Strep-Gal, GIBCO-BRL) is employed. The membrane is incubated with a 3-ml (option) mixture containing 700 X diluted Strep-Gal (1.38 units/ml, enzyme activity), 4% polyethylene glycol 8000 (Sigma, St. Louis, MO), and 0.3% BSA in 1 X TBS buffer for 2 hours. The membrane is then washed with 1 X TBS buffer three times for 10 min each. The chromogen is generated by treating the membrane with X-gal substrate containing 1.2 mM X-gal, 1

mM $MgCl_2$, 3 mM $K_3Fe(CN)_6$, 3 mM $K_4Fe(CN)_6$ in 1 X TBS buffer for 30 ~ 60 minutes at 37 °C for β-galactosidase reaction. The color development reactions are then stopped by 1x PBS containing 20 mM EDTA. After color development the cDNA molecules labeled with biotin yield a blue chromogen. To measure the expression levels of the genes, the membrane is scanned or taken image by a flatbed scanner (PowerLook 3000). The scanner provided 3000 dpi resolution and is suitable for larger arrays such as arrays of 9600 elements.

Molecular Cloning and Plasmid Constructs

Total RNA is reverse transcribed by using SuperScript II RTase (Gibco-BRL, Rockville, Maryland) and random hexamer. A cDNA encoding the entire interesting gene coding region is amplified from the cDNA of PC12 by PCR. The reaction mixture is denatured at 94 ℃ for 30 s, annealed at 60 ℃ for 30 s, and extended at 72 ℃ for 1min 30s. These reactions are repeated for 30 cycles. The interesting cDNA fragment is digested by restriction enzymes and cloned into a pTRE2 vector according to the manufacturer's instructions.

RNA Isolation and Northern Blot Analysis

Total RNA is isolated from cardiomyocytes by the guanidine isothiocyanate/phenol chloroform method. The RNA (10 μg/lane) is separated by electrophoresis on a 1% agarose formaldehyde gel and transferred onto a nylon membrane (Nytran, Schleicher & Schuell, Inc) by a vacuum blotting system (VacuGene XL, Pharmacia). After hybridization with the ^{32}P-labeled cDNA probes, the membrane is washed with 1x SSC containing 1% SDS at room temperature for 30 min and then exposed to x-ray film at -70°C. Autoradiographic results are analyzed by using a densitometer (Computing Densitometer 300S, Molecular Dynamics).

Western Blot Analysis

Western blot analysis is performed as previously described [21]. Briefly, cardiomyocytes are lysed with buffer containing 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, and a protease inhibitor mixture (PMSF, aprotinin, and sodium orthovanadate). For detection of eNOS protein in cardiomyocytes, the cell lysates are collected and boiled. Total cell lysates (100 μg of protein) are separated by SDS-PAGE (12% running, 4% stacking) and transferred onto a polyvinylidene fluoride membrane (Immobilon P, 0.45-μm pore size). The membrane is then incubated with specific antibody. Immunodetection was performed by using the Western-Light chemiluminescent detection system (Tropix, Inc).

Measurement of NOS Activity

To measure NOS activity, L-arginine to L-citrulline conversion is assayed in cardiomyocytes with the NOS detection assay kit (Calbiochem) according to the manufacturer's instructions. Briefly, cardiomyocytes are lysed with buffer containing 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS. After incubation on ice for 30 min, cell extracts are centrifuged to remove cell debris. Protein extracts are then incubated for 60 min at 37 ^{o}C in a solution of 10 mmol/l L-[^{14}C]arginine, 1 mmol/L NADPH, 1mol/L FAD, 1 mol/L FMN, 100 nmol/L calmodulin, 600 mol/L $CaCl_2$, and 3 mol/L ahydrobiopterin in a final volume of 40 μl. The reaction is stopped by the addition of 400 μl of stop buffer (10 mmol/L EDTA, 50 mmol/L HEPES buffer, pH 5.5) to the reaction mixture. Then 100 μl of equilibrated resin is added to each mixture. Reaction samples are transferred to spin cups and centrifuged at 10,000g for 30 s. Radioactivity of the flow through is measured by liquid scintillation counting. Enzyme activity is expressed as citrulline production in pmol/min/mg protein.

Measurement of Nitrate/Nitrite Levels

The culture medium is stored at –70 ºC until use. After the medium had been thawed, the sample is deproteined with two volumes of 4°C 99% ethanol and centrifuged (3000g for 10 min). These medium samples (100 ml) are injected into a collection chamber containing 5 % VCl_3. This strong reducing environment converts both nitrate and nitrite to NO. A constant stream of helium gas carried NO into a NO analyzer (Seivers 270B NOA; Seivers Instruments Inc., Boulder, CO), where the NO is reacted with ozone, resulting in the emission of light. Light emission is proportional to the NO formed; standard amounts of nitrate are used for calibration.

Protein Synthesis Measurement (^{3}H-Leucine Incorporation)

To measure synthesis of new protein, cardiomyocytes cultured on the flexible membrane base culture dish are incubated with 1.0 μCi/ml ^{3}H-leucine in serum-free medium. After addition of agent indicated, cells are harvested by incubation at 4 ℃ with trichloroacetic acid (5%) followed by solubilization in 0.1 N NaOH, and radioactivity is determined by scintillation counting.

Electrophoretic Mobility Shift Assay

The electrophoretic mobility shift assay is performed as previously described (Wung et al., 1997). To prepare nuclear protein extracts, cardiomyocytes are washed with cold PBS and then immediately removed by scraping in PBS. After centrifugation of the cell suspension at 2000 rpm, the cell pellets are resuspended in cold buffer A (containing, in mmol/L, KCl 10, EDTA 0.1, DTT 1, and PMSF 1) for 15 min. The cells are lysed by adding 10% NP-40 and then centrifuged at 6000 rpm to obtain pellets of nuclei. The nuclear pellets are resuspended in cold buffer B (containing, in mmol/L, HEPES 20, EDTA 1, DTT 1, and PMSF 1, and 0.4 mol/L NaCl), vigorously agitated, and then centrifuged. The supernatant containing the

nuclear proteins is used for the assay or stored at -70°C until used. Double-stranded oligonucleotides (30 bp) containing specific transcriptional factor binding site are synthesized and annealed. The oligonucleotides are end labeled with [^{32}P]ATP. Extracted nuclear proteins (10 μg) are incubated with 0.1 ng ^{32}P-labeled DNA for 15 min at room temperature in 25 μL binding buffer containing 1 μg poly (dI-dC). In the antibody competition assay, specific antibodies are incubated with the mixture for 10 min at room temperature followed by the addition of the labeled probe. The mixtures are electrophoresised on 5% non-denaturing polyacrylamide gels. Gels are dried and imaged by autoradiography.

Statistical Analysis

Results are expressed as mean ± S.E.M of at least three experiments unless designated otherwise. Statistical analysis was performed using analysis of variance and Student's t test as appropriate. A value of $p<0.05$ was considered to be statistically significant.

第三章 Differential expression of mechanical stretch-induced endothelial nitric oxide synthase gene in neonatal rat cardiomyocytes by cDNA microarray

中文摘要

生物物理壓力如機械張力(mechanical stretch)的作用於心臟細胞，會導致心臟細胞的肥大現象，對臨床及基礎的心臟學研究而言，如何控制心臟細胞肥大現象的發生，是一個具有挑戰性的問題，尤其對發展新的心臟疾病治療策略更為重要。至目前為止，控制心臟細胞肥大現象的發生的機制仍不清楚，而回答此問題的一個方法就是找出控制心臟細胞肥大現象的發生的一系列基因。假如我們能明瞭如何控制心臟細胞肥大現象發生的機制，或許就能抑制心臟細胞肥大現象的發生，甚至後續的細胞凋亡，進而發展出新的心臟衰竭或心臟相關疾病的治療策略。為達此目標，篩選及定性參與心臟細胞肥大現象的發生的關鍵分子是必要的。以機械張力作用於心臟細胞，造成心臟細胞的肥大現象，對於細胞內不同基因表現的影響以及相關基因功能的闡釋尚有許多不明之處。在本研究計劃中，我們將運用基因微陣列（cDNA microarray）分析法探討機械張力對心臟細胞基因表現的作用。基因微陣列為發展技術較成熟且能同時分析大量基因的科技之一，已廣泛應用於許多研究領域以鑑定新的基因。

在本計劃中，我們將以機械張力作用於細胞能誘發心臟細胞肥大的實驗模式，觀察機械張力對心臟細胞的作用，並利用微陣列剖繪其基因表現的型態。將所篩選的基因送入原細胞中並大量表現，研究其對機械張力所誘發心臟細胞肥大的調控作用，配合微陣列可研究其下游受影響的基因為何。為研究基因的調控作用，將選殖啟動子並調查甲基化對細胞肥大的影響。對篩選出的基因，進一步將研究其蛋白質間的交互作用及在細胞中的作用位置，這些努力將提供許多的線索以進一步闡釋心臟細胞肥大的分子機制，並有助於發展新的治療策略。

以基因微陣列分析機械張力對心臟細胞的作用，驗結果顯示：機械張力可以影

響心臟細胞內許多基因的表現(表一) ，其中包括增加內皮素型一氧化氮合成酶(endothelial nitric oxide synthase; eNOS)的基因表現，此增加作用可以北方式點墨法及西方式點墨法作進一步的確認，機械張力亦會短暫增加 eNOS 的酵素活性及一氧化氮的生成，若給予心臟細胞一氧化氮供獻劑如 S-nitroso-N-acetylpenicillamine (SNAP)或 3-morpholinosydnonimine (SIN)則能抑制機械張力誘發心臟細胞肥大的作用，由此初步結果可知機械張力會短暫增加 eNOS 的基因表現，並於機械張力誘發心臟細胞肥大的過程中扮演保護的作用，進一步的研究工作將探討機械張力對心臟細胞其它基因表現的作用並設計實驗闡釋這些相關基因的生理功能。

關鍵詞： 機械張力 •基因微陣列• 內皮素型一氧化氮合成酶 • 心臟細胞肥大

英文摘要

Mechanical stretch induced by high blood pressure is an initial factor leading to cardiac hypertrophy. However, the effect of mechanical stretch on gene induction in cardiomyocytes still remains unclear. In the present study, we compared the transcript profiles of mechanically-stretched neonatal rat cardiomyocytes with that of untstretched cells by hybridization of cell-derived cDNA to DNA probes immobilized on microarrays. Neonatal rat cardiomyocytes were cultured on malleable silicone dishes and were stretched by 20%. We compared the transcript profiles of cardiomyocytes under mechanical stretch for 60 minutes with that of stationary control cells by hybridization of cell-derived cDNA to DNA probes immobilized on microarrays. The microarrays contained probes for 480 known genes including signal transduction, cell cycle regulators, cytoskeleton and cell motility, and so on. Eighteen genes were identified that showed significantly differential expression in response to mechanical stretch in cardiomyocytes. Of the represented genes expressed, endothelial nitric oxide synthase (eNOS) gene was the most interesting one. Northern blot and western blot

analysis further quantified the expression of eNOS gene. Mechanical stretch also increased constitutive NOS activity and NO production. Furthermore, NO donors such as S-nitroso-N-acetylpenicillamine (SNAP) or 3-morpholinosydnonimine (SIN) inhibited the mechanical stretch-induced cardiomyocyte hypertrophy. Our results indicate that mechanical stretch transiently induces eNOS gene expression, thereby increasing constitutive NOS activity and NO production, which plays a protective role in the prevention of cardiomyocyte hypertrophy.
Keywords: mechanical stretch •cDNA microarray •endothelial nitric oxide synthase • cardiomyocytes •rat

前言

Functional genome analysis is thought to be one of the most promising research projects in twenty-first century after human genome project. Some new and foresighted gene analysis techniques were developed in the latter of the nineties, such as cDNA microarray[1, 2], DNA chip[3], serial analysis of gene expression[4] and others. cDNA microarray is the more mature technique among them, and have been applied in various biological studies for identifying differentially expressed genes.[2, 5-7] Microarray method can be used either for monitoring the expression levels of known genes or for searching differentially expressed genes. Especially after large amounts of ESTs are published,[8] it will be even more powerful in its detection of abnormal genes for diseases, e.g., cancer and genetic diseases.

研究目的

In the present study, we compared the transcript profiles of mechanically-stretched neonatal rat cardiomyocytes with that of stationary control cells by hybridization of cell-derived cDNA to DNA probes immobilized on microarrays. The microarrays

contained probes for 480 known genes. We found that endothelial nitric oxide synthase (eNOS) gene was induced by mechanical stretch. The expression of eNOS gene was further quantified by Northern blot and western blot analysis. Mechanical stretch also increased constitutive NOS activity and NO production.

文獻探討

Mechanical stretch has been reported to cause an induction of immediate-early genes such as *c-fos*,[9] *c-jun*, *c-myc* and *egr-1*[10] and increase several gene expression such as atrial natriuretic peptide[11] and endothelin-1[12] in cardiomyocytes. Our previous studies also demonstrated that mechanical stretch induces angiotensinogen gene expression and regulates myosin heavy chain gene expression in cardiomyocytes.[13, 14] However, the effect of mechanical stretch on gene induction in cardiomyocytes still remains unclear. This report clearly shows for the first time that mechanical stretch induces eNOS gene expression, thereby increasing constitutive NOS activity and NO production in cardiomyocytes. Furthermore, NO donors such as S-nitroso-N-acetylpenicillamine (SNAP) or 3-morpholinosydnonimine (SIN) inhibited the mechanical stretch-induced cardiomyocyte hypertrophy. Our results indicate that mechanical stretch transiently induces eNOS gene expression, thereby increasing constitutive NOS activity and NO production, which plays a protective role in the prevention of cardiomyocyte hypertrophy.

結果與討論

Results

Microarray analysis of stretch-induced gene expression in cardiomyocytes.

In comparing control and stretched cardiomyocytes gene expression for the 480 known genes, 172 cDNAs had a detectable signal in each of groups (Figure 1A). The differentially expressed genes were chosen beyond the 95 % confidence interval (C.I.)

of prediction lines (the dot curve in Figure 1 B). Eighteen genes were differentially expressed in both samples. Of these, only ten were induced after mechanical stretch; in contrast, eight EST clones were decreased by mechanical stretch. These genes may relate to cell growth and cell cycle, hormone or cytokine, stress-response, signal transduction, cell surface antigen/cell adhesion, metabolism, transcription factors, and so on.

Stretch induces eNOS gene expression in cardiomyocytes.

We next investigated whether mechanical stretch induces increase in eNOS mRNA and protein levels in cardiomyocytes. As shown in Figure 2A, unstimulated and stretched cardiomyocytes both expressed detectable eNOS mRNA; however, stretched cardiomyocytes clearly exhibited a stronger signal within a period of 2 hours, which then declined gradually. Densitometric analysis of eNOS gene expression normalized with 18S showed a 1.4±0.4–fold increase in cardiomyocytes exposed to mechanical stretch for one hour compared with control cells (n=4). To ascertain the significance of increased eNOS gene expression, eNOS protein levels were examined by Western blotting analysis in cardiomyocytes that were subjected to mechanical stretch for different time interval. Immunoreactive eNOS was detected in unstimulated and stretched cardiomyocytes (Figure 2B). The eNOS protein band, with a molecular mass of 140 kDa increased after exposure to the mechanical stretch for one hour, and then these eNOS protein accumulations were returned to baseline within 12 hours. There was an increase in eNOS protein (1.3±0.3-fold, n=3) after 2 hours of mechanical stretch compared with stationary controls.

Effects of mechanical stretch on NO synthesis and NOS activity.

We further investigated the effect of the mechanical stretch on NOS activity and NO production in cardiomyocytes. As shown in Figure 3A, mechanical stretch induced

eNOS enzyme activity within 2 hours, as measured by conversion of [^{14}C]L-arginine to [^{14}C]L-citrulline. Maximal effects occurred after 1- and 2-hour of mechanical stretch (ie, 1.9- and 2.1-fold increases respectively compared with stationary controls). Similarly, treatment of cardiomyocytes with mechanical stretch also increased NO production in cultured cardiomyocytes within 2 hours (Figure 3B). Treatment of cardiomyocytes with mechanical stretch for 2 hours caused a 2.8–fold increases in accumulation of nitrite and nitrate in the culture medium.

Effect of NO donors on mechanical stretch-induced protein synthesis

The effect of mechanical stretch on protein synthesis was analyzed by measurement of ^{3}H-leucine incorporation into the myocytes. Mechanical stretch increased ^{3}H-leucine incorporation in myocytes (Figure 4A). The effects of NO donors such as S-nitroso-N-acetylpenicillamine (SNAP) and 3-morpholinosydnonimine (SIN) or NO scavenger 2-phenyl-4,4,5,5,-tetramethyl-imidazoline-l-oxyl-3-oxide (PTIO) on the stretch-increased protein synthesis were investigated (Figure 4B). Pretreatment with SNAP (100 μM) or SIN (100 μM) for 30 min significantly inhibited the stretch-increased ^{3}H-leucine incorporation, indicating that NO might inhibit mechanical stretch-induced increase of protein synthesis in cardiomyocytes.

Discussion

In the present study, we applied cDNA microarray expression profiling to study the effects of mechanical stretch on cardiomyocytes. Gene expression differences during mechanical stretch treatment as summarized in Table 1 are newly associated with mechanical stretch. However, these results are not quite comprehensive even for the genes on our microarray. Signal dilution due to species–specific expression could cause a signal below the differential expression threshold of detection. Our data on changes in gene expression after mechanical stretch treatment illustrate how

microarrays may be used to monitor the progression of the cell molecular phenotype and, as a discovery tool, to identify new genes associated with myocardial stress. This technology should be a potent hypothesis generator in experiments designed to elucidate the mechanisms of disease pathogenesis and to determine the basis of drug action.

Cyclic mechanical stretch and shear stress represent important components of the mechanical environment that regulate gene expression in endothelium, vascular smooth muscle, and myocardium. In bovine aortic endothelial cells, cyclic mechanical stretch augments endothelial NOS expression and NO production.[18] In human aortic endothelial cells, pulsatile stretch increases both endothelial NOS protein and mRNA expression.[19] Mechanical stretch also stimulates nitric oxide production by rapid activation of eNOS in adult rat cardiomyocytes.[20] In this study, our finding that mechanical stretch induces eNOS gene expression, thus increasing constitutive NOS activity and NO production in cardiomyocytes are consistent with those studies. However, Yamamoto *et al* reported that mechanical strain failed to increase NO production in cardiomyocytes.[21] There are several possible explanations for this discrepancy. One of the most plausible reasons is the difference in the method used to measure the NO concentration. In this study, we measured the NO concentration by using the Sievers NO analyzer that has much higher sensitivity than the conventional Griess reaction method.

Accumulating evidences indicate that NO or related molecules modulate cellular responses. NO exerts its action by stimulating soluble guanylate cyclase, leading to an increase of cyclic GMP levels that activate cyclic GMP-dependent protein kinases.[22] In the present study, it is shown that mechanical stretch induces eNOS expression and NO production in cardiac myocytes. These findings suggest that mechanical stretch may

play an important role in eNOS expression, which provides preventive effect in the initial state of the pathogenesis of cardiomyocytes hypertrophy. This study also demonstrates the potential of cDNA microarray as a critical tool in functional genome analysis.

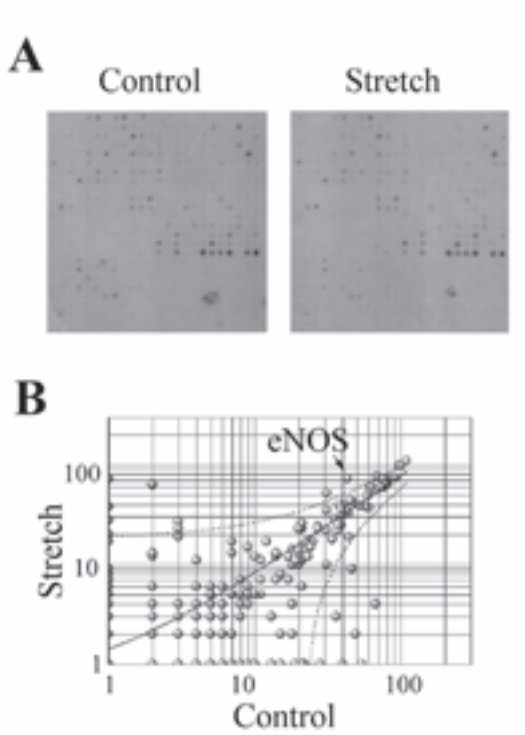

Figure 1. cDNA microarray analysis of mechanical stretch-induced gene expression in neonatal rat cardiomyocytes. (A) Expression profiling of mechanical stretch induced genes in neonatal rat cardiomyocytes. The mRNAs of control (left panel) and stretch-treated neonatal rat cardiomyocytes (right panel) respectively are used to perform microarray analysis for 480 known genes. (B) The outliers of microarray analysis. The differentially expressed genes beyond the 95 % confidence interval of prediction lines (dot curves; while the solid line denotes the regression line) together with a stretch to control expression ratio of more than 1.5 are selected as the candidates. The endothelial nitric oxide synthase is one of the mechanical stretch-induced genes.

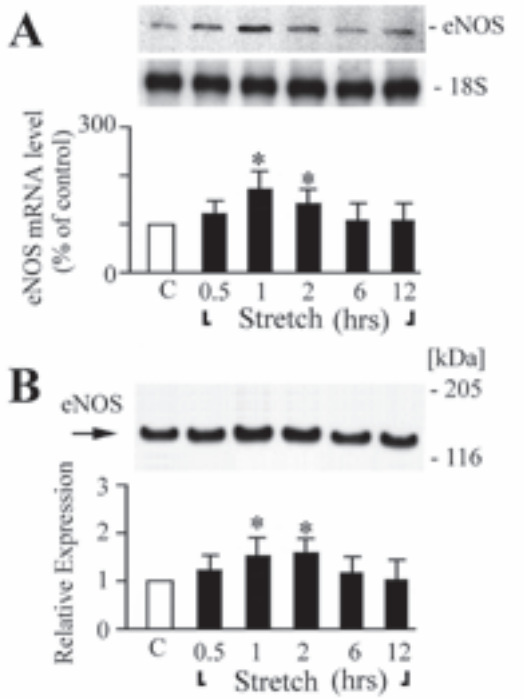

Figure 2. Stretch induces eNOS gene expression in cardiomyocytes. (A) Time course of mechanical stretch on eNOS mRNA expression. Cells were stretched for the indicated times. Total RNA was extracted and Northern hybridization was performed with ^{32}P-labeled rat eNOS as probe. 18S RNA was used to normalize the RNA applied in each lane. Data was presented as a percentage change of experimental groups compared to untreated controls. Results are shown as mean $\pm$ SEM from four independent experiments. *P<0.05 vs control. (B) Effects of mechanical stretch on eNOS protein expression. Cardiomyocytes were exposed to mechanical stretch for various time periods. Cell extracts were subjected to SDS-polyacrylamide gel electrophoresis, followed by immunoblot analysis using anti-eNOS antibody. The molecular mass of eNOS protein is 140 kDa. Data was presented as a percentage change of experimental groups compared to untreated controls. Results are shown as mean $\pm$ SEM from three independent experiments. *P<0.05 vs control.

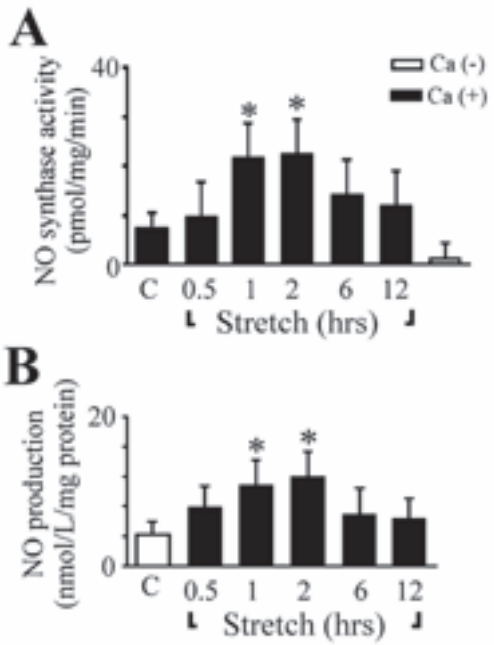

Figure 3. Stretch increases NOS activity and NO production in cardiomyocytes. (A) Stretch increases NO synthase activity in cardiomyocytes. NOS activities were measured by citrulline formation from cell lysate incubated with or without Ca^{2+}. NOS activity in the absence of Ca^{2+} indicates inducible NOS activity, whereas its activity in the presence of Ca^{2+} represents constitutive NOS activity. Cardiomyocytes were treated with mechanical stretch for 0.5, 1, 2, 6 or 12 hours. Results are shown as mean $\pm$ SEM from three independent experiments. *P<0.05 vs control. (B) Time course of stretch-increased NO production in cardiomyocytes. Cardiomyocytes were in control condition (C), treated with mechanical stretch for 0.5, 1, 2, 6 or 12 hours. Results are shown as mean $\pm$ SEM from five independent experiments. *P<0.05 vs control.

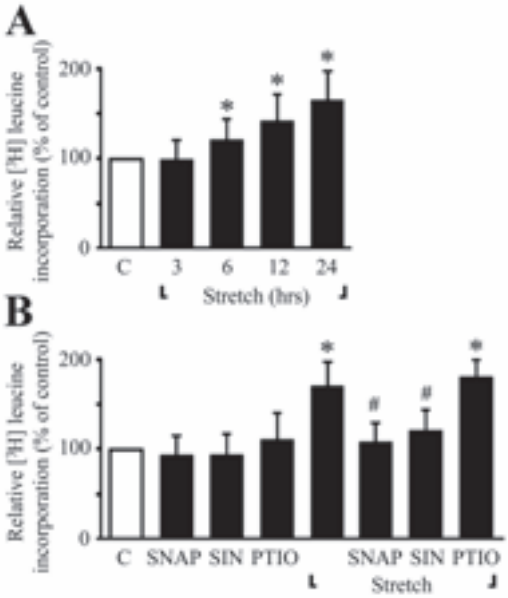

Figure 4. Amino acid uptake by cardiac myocytes. (A) Time course of stretch-increased amino acid uptake in cardiomyocytes. Cardiomyocytes were in control condition (C), treated with mechanical stretch for 3, 6, 12 or 24 hours. Results are presented as mean $\pm$ SEM from three independent experiments. (B) After treatment with SNAP and SIN or PTIO, cardiac myocytes were stretched by 20% for 24 hours. Results are shown as mean $\pm$ SEM from five independent experiments. The relative amount of protein synthesis was determined by assessing incorporation of the radioactivity into a trichloroacetic acid-insoluble fraction. *P<0.05 compared with unstretched control. #P<0.05 compared with stretched cardiomyocytes without SNAP and SIN or PTIO.

第四章 Differential expression of mechanical stretch-induced activator protein-2 gene in neonatal rat cardiomyocytes by cDNA microarray

中文摘要

機械張力(mechanical stretch)的作用於心臟細胞，會導致心臟細胞的肥大現象，對臨床及基礎的心臟學研究而言，如何控制心臟細胞肥大現象的發生，是一個具有挑戰性的問題，尤其對發展新的心臟疾病治療策略更為重要。至目前為止，控制心臟細胞肥大現象的發生的機制仍不清楚，而回答此問題的一個方法就是找出控制心臟細胞肥大現象的發生的一系列基因。假如我們能明瞭如何控制心臟細胞肥大現象發生的機制，或許就能抑制心臟細胞肥大現象的發生，甚至後續的細胞凋亡，進而發展出新的心臟衰竭或心臟相關疾病的治療策略。為達此目標，篩選及定性參與心臟細胞肥大現象的發生的關鍵分子是必要的。以機械張力作用於心臟細胞，造成心臟細胞的肥大現象，對於細胞內不同基因表現的影響以及相關基因功能的闡釋尚有許多不明之處。在本研究計劃中，我們運用基因微陣列（cDNA microarray）分析法探討機械張力對心臟細胞基因表現的作用。基因微陣列為發展技術較成熟且能同時分析大量基因的科技之一，已廣泛應用於許多研究領域以鑑定新的基因。

在本計劃中，我們以機械張力作用於細胞能誘發心臟細胞肥大的實驗模式，觀察機械張力對心臟細胞的作用，並利用微陣列剖繪其基因表現的型態。將所篩選的基因送入原細胞中並大量表現，研究其對機械張力所誘發心臟細胞肥大的調控作用，配合微陣列可研究其下游受影響的基因為何。

以基因微陣列分析機械張力對心臟細胞的作用，第二年的實驗結果顯示：機械張力可以影響心臟細胞內許多基因的表現，其中包括增加轉錄因子活化蛋白2(activator protein-1; AP-2)的基因表現，此增加作用可以北方式點墨法作進一步的確認，由此初步結果可知機械張力會短暫增加轉錄因子活化蛋白 2 的基因表

現，進一步的研究工作將探討機械張力對心臟細胞其它基因表現的作用並設計實驗闡釋這些相關基因的生理功能。

關鍵詞：機械張力、基因微陣列、心臟細胞肥大

Abstract

Mechanical stretch induced by high blood pressure is an initial factor leading to cardiac hypertrophy. However, the effect of mechanical stretch on gene induction in cardiomyocytes still remains unclear. In the present study, we compared the transcript profiles of mechanically-stretched neonatal rat cardiomyocytes with that of untstretched cells by hybridization of cell-derived cDNA to DNA probes immobilized on microarrays. Neonatal rat cardiomyocytes were cultured on malleable silicone dishes and were stretched by 20%. We compared the transcript profiles of cardiomyocytes under mechanical stretch for 60 minutes with that of stationary control cells by hybridization of cell-derived cDNA to DNA probes immobilized on microarrays. The microarrays contained probes for 480 known genes including signal transduction, cell cycle regulators, cytoskeleton and cell motility, and so on. Eighteen genes were identified that showed significantly differential expression in response to mechanical stretch in cardiomyocytes. Of the represented genes expressed, activator protein-2 (AP-2) gene was the most interesting one. Northern blot and western blot analysis further quantified the expression of AP-2 gene. Our results indicate that mechanical stretch transiently induces AP-2 gene expression. Further study will be performed to unravel the role of AP-2 in the process of cardiomyocyte hypertrophy.

Keywords: Mechanical Stretch, cDNA Microarray, Cardiomyocyte Hypertrophy

前言

Functional genome analysis is thought to be one of the most promising research projects in twenty-first century after human genome project. Some new and foresighted

gene analysis techniques were developed in the latter of the nineties, such as cDNA microarray[1, 2], DNA chip[3], serial analysis of gene expression[4] and others. cDNA microarray is the more mature technique among them, and have been applied in various biological studies for identifying differentially expressed genes.[2, 5-7] Microarray method can be used either for monitoring the expression levels of known genes or for searching differentially expressed genes. Especially after large amounts of ESTs are published,[8] it will be even more powerful in its detection of abnormal genes for diseases, e.g., cancer and genetic diseases.

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文獻探討

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AP-2 in the process of cardiomyocyte hypertrophy.

結果與討論

Results

Microarray analysis of stretch-induced gene expression in cardiomyocytes.

In comparing control and stretched cardiomyocytes gene expression for the 480 known genes, 172 cDNAs had a detectable signal in each of groups (Figure 1A). The differentially expressed genes were chosen beyond the 95 % confidence interval (C.I.) of prediction lines (the dot curve in Figure 1 B). Eighteen genes were differentially expressed in both samples. Of these, only ten were induced after mechanical stretch; in contrast, eight EST clones were decreased by mechanical stretch. These genes may relate to cell growth and cell cycle, hormone or cytokine, stress-response, signal transduction, cell surface antigen/cell adhesion, metabolism, transcription factors, and so on.

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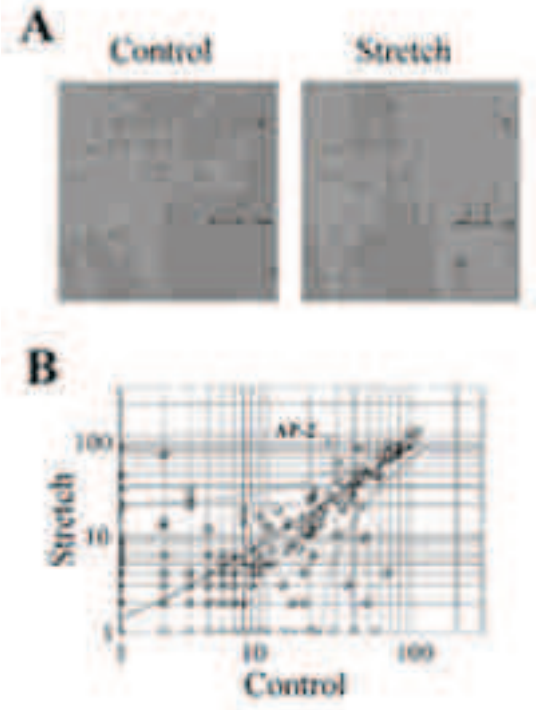
Discussion

In the present study, we applied cDNA microarray expression profiling to study the effects of mechanical stretch on cardiomyocytes. Our data on changes in gene expression after mechanical stretch treatment illustrate how microarrays may be used

to monitor the progression of the cell molecular phenotype and, as a discovery tool, to identify new genes associated with myocardial stress. This technology should be a potent hypothesis generator in experiments designed to elucidate the mechanisms of disease pathogenesis and to determine the basis of drug action.

Cyclic mechanical stretch and shear stress represent important components of the mechanical environment that regulate gene expression in endothelium, vascular smooth muscle, and myocardium. In the present study, it is shown that mechanical stretch induces AP-2 expression in cardiac myocytes. Recently, we have demonstrated that the existence of specific upstream sequence between –273 and –188 bp required for induction of β-myosin heavy chain gene by endothelin-1 (ET-1) corresponds to the region which contains a AP-2 motif at –216 to –208 bp (GCCCCCTGGA) (Figure 3). Furthermore, as shown in Figure 4, the ability of ET-1 to increase the binding of cardiomyocyte nuclear extracts to the AP-2 consensus binding sequence oligonucleotide were inhibited by antioxidant N-acetylcysteine (NAC). These results suggest that the AP-2 binding site could be the ET-1-responsive *cis*-acting regulatory element(s). However, the molecular mechanism of AP-2 in the process of cardiomyocyte hypertrophy remains to be investigated. These findings suggest that mechanical stretch may play an important role in AP-2 expression, which involves in the initial state of the pathogenesis of cardiomyocytes hypertrophy. This study also demonstrates the potential of cDNA microarray as a critical tool in functional genome analysis.



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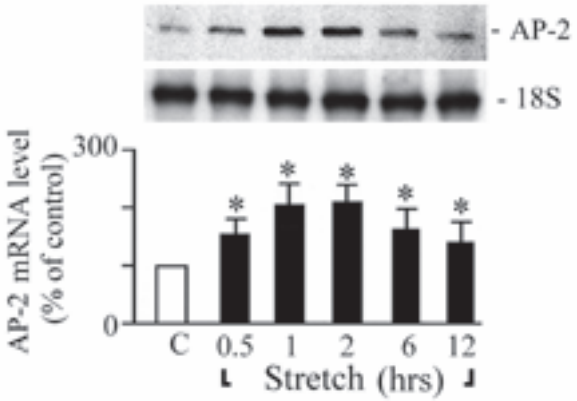

Figure 2. Stretch induces AP-2 gene expression in cardiomyocytes. Time course of mechanical stretch on AP-2 mRNA expression. Cells were stretched for the indicated times. Total RNA was extracted and Northern hybridization was performed with ^{32}P-labeled rat AP-2 as probe. 18S RNA was used to normalize the RNA applied in each lane. Data was presented as a percentage change of experimental groups compared to untreated controls. Results are shown as mean ± SEM from four independent experiments. *P<0.05 vs control.

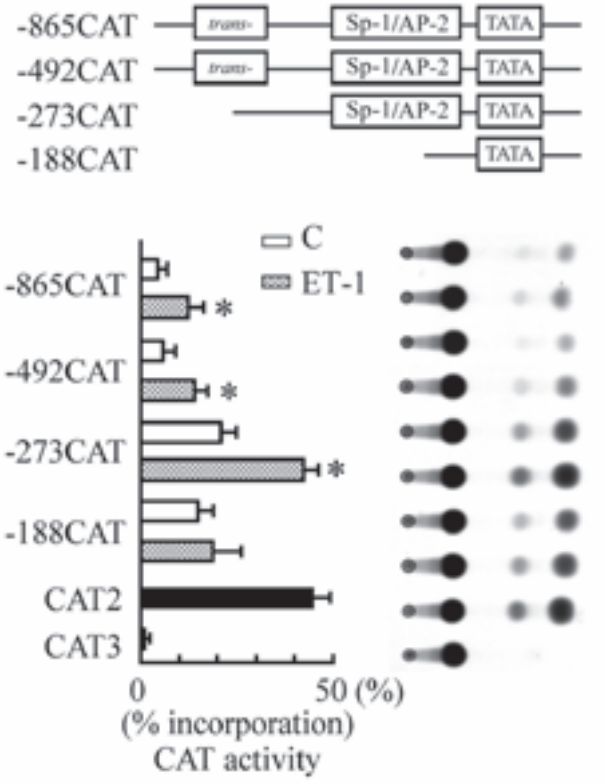

Figure 3. Localization of the β-MyHC promoter region in response to ET-1. Cardiomyocytes were transiently transfected with the β-MyHC CAT constructs and treated with 10nM ET-1 for 48h as described before. Results are shown as means ± SEM from four separate experiments. *P<0.05 vs. control.

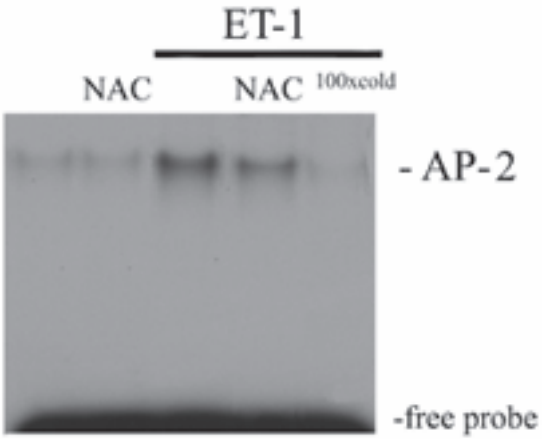

Figure 4. NAC attenuated the ET-1–stimulated AP-2 binding activity in cardiomyocytes. This binding activity was measured by using the electrophoretic mobility shift assay. Cells were preincubated with NAC (10mM) for 30min after incubation with ET-1 (10nM) for 6h. *100 x Cold* denotes a 100-fold molar excess of unlabeled oligonucleotide relative to the radiolabeled probe; this was added to the binding assay for competition with the unlabeled oligonucleotide. The experiment was repeated 2 times, with reproducible results.

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第六章 計畫成果自評：

a. 研究內容與原計畫完全相符、順利達成預期目標、研究成果之學術價值甚高、適合在學術期刊發表。

b. 計劃經費補助已發發表相關著作:

1. Chen HH, Hong HJ, Chou YH, **Cheng TH**, Chen JJ, Lin H. Inhibition of Cyclic Strain-induced Endothelin-1 Secretion by Baicalein in Human Umbilical Vein Endothelial Cells. ***Planta Medica*** (in press)
2. Yang HY, Liu JC, Chen YL, Chen CH, Lin H, Lin JW, Chiu WT, Chen JJ, **Cheng TH***. (2005) Inhibitory effect of trilinolein on endothelin-1-induced *c-fos* gene expression in cultured neonatal rat cardiomyocytes. ***Naunyn-Schmiedeberg's Archives of Pharmacology*** 372(2):160-167. ***(*Corresponding Author)***
3. Bi WF, Yang HY, Liu JC, **Cheng TH**, Chen CH, Shih CM, Lin H, Wang TC, Lian WS, Chen JJ, Chiu HC, Chang NC. (2005) Inhibition of cyclic strain-induced endothelin-1 secretion by tetramethylpyrazine. ***Clinical and Experimental Pharmacology and Physiology***. 32(7):536-540.
4. **Cheng TH**, Shih NL, Chen CH, Lin H, Liu JC, Chao HH, Liou JY, Chen YL, Tsai HW, Chen YS, Cheng CF, Chen JJ (2005) Role of mitogen-activated protein kinase pathway in reactive oxygen species-mediated endothelin-1-induced β-myosin heavy chain gene expression and cardiomyocyte hypertrophy. ***Journal of Biomedical Science*** 12(1):123-33.
5. Juan SH, Chen JJ, Chen CH, Lin H, Cheng CF, Liu JC, Hsieh MH, Chen YL, Chao HH, Chen TH, Chan P, **Cheng TH*** (2004) 17β-Estradiol inhibits cyclic strain-induced endothelin-1 gene expression within vascular endothelial cells. ***American Journal of Physiology- Heart and Circulatory Physiology*** 287(3):H1254-61. ***(*Corresponding Author)***

6. **Cheng TH**, Liu JC, Lin H, Shih NL, Chen YL, Huang MT, Chan P, Cheng CF, Chen JJ. (2004) Inhibitory effect of resveratrol on angiotensin II-induced cardiomyocyte hypertrophy. ***Naunyn-Schmiedeberg's Archives of Pharmacology*** 369: 239-244.
7. Liu JC, Chan P, Chen JJ, Lee HM, Lee WS, Shih NL, Chen YL, Hong HJ, **Cheng TH*** (2004) The inhibitory effect of trilinolein on norepinephrine-induced β–myosin heavy chain promoter activity, reactive oxygen species generation, and extracellular signal-regulated kinase phosphorylation in neonatal rat cardiomyocytes. ***Journal of Biomedical Science*** 11: 11-18. ***(*Corresponding Author)***
8. Liu JC, Chen JJ, Chan P, Cheng CF, **Cheng TH*** (2003) Inhibition of cyclic strained-induced endothelin-1 gene expression by resveratrol. ***Hypertension*** 42: 1198-205. ***(*Corresponding Author)***